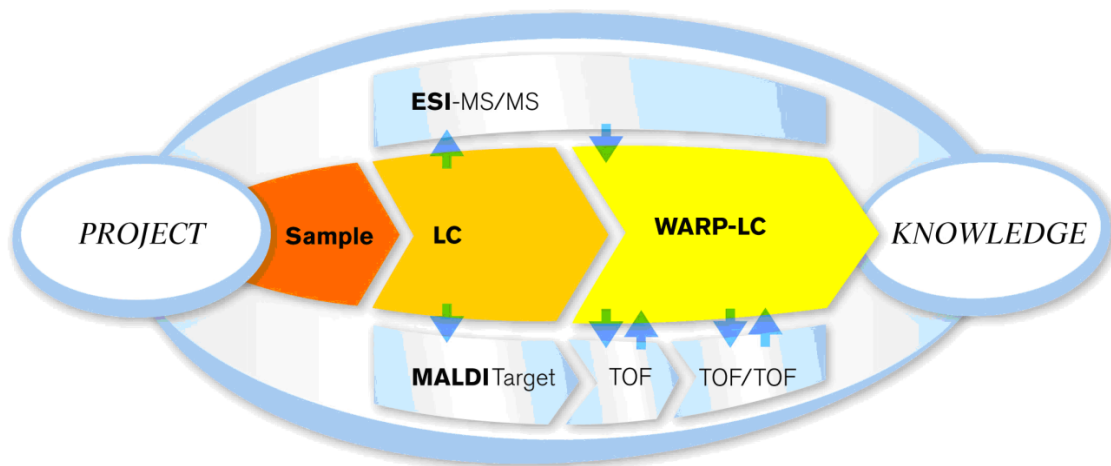




LC-MALDI Analysis of a ICPL-labeled Protein Digest Mixture



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Contact

Contact your local Bruker Daltonics representative for service and further information.

USA

Bruker Daltonics Inc
40 Manning Road
Billerica, MA 01821
USA
Phone: +1 978 6633660
Fax: +1 978 6675993
Internet: www.bdal.com

Service Support

Phone: +1 978 6633660-1445
E-mail: service-support@bdal.com

Germany

Bruker Daltonik GmbH
Fahrenheitstraße 4
28359 Bremen
Germany
Phone: +49 421 2205-450
Fax: +49 421 2205-370
Internet: www.bdal.de

Service Support

Phone: +49 421 2205-350
E-mail: service@bdal.de

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Introductory Remarks

Related software or higher:

Compass 1.3 Patch 5 for flexSeries (flexControl; flexAnalysis)

Mascot 2.3

ProteinScape 3.0

Note In contrast to Warp-LC 1.2, this new Warp-LC version does not support BioTools for database searches. All searches have to be performed with ProteinScape 3.0!

This tutorial describes the setup of a LC-MALDI SILE analysis for peptide quantification of a low complex protein sample. Exemplary shown in this tutorial is the LC-MALDI SILE analysis of a 3 protein digest mixture labeled with the ICPL-duplex kit. The data set of this

LC-MALDI analysis is available as tutorial data set “LC-MALDI-SILE” ’ on the WARP-LC installation DVD.

1 Introduction

1.1 General

In **S**table **I**sotope **L**abeling **E**xperiments (SILE), quantitative information about proteins in complex mixtures is obtained. All current protein and peptide label chemistries such as ICPL, iTRAQ, SILAC, ICAT, and ^{18}O -C-terminal labeling can be used in this workflow using WARP-LC 1.3. Typically 2 states of a proteome or a protein/peptide mixture are compared in SILE. If more than two mixtures are compared in a single LC-MS/MS experiment it is called multiplex-SILE.

Each mixture is modified with a different isotopomer of the labeling reagent. Identical proteins or peptides that are present in each mixture can now be distinguished in an MS or MS/MS measurement and subjected to relative quantification. The mixtures are combined and typically analyzed by LC-MS/MS. As one of the isotopomers acts as an internal standard, relative quantification can be achieved with high accuracy using WARP-LC 1.3. Current label chemistries provide technical quantification errors of less than 10–20%, which is a lot better than the typical biological variation in these experiments.

Using a non-isobaric label like ICPL or SILAC, the quantitative information is read out from the MS data and MS/MS is used to identify those peptides that are regulated. Using an isobaric label like iTRAQ, all MS-peaks need to be MS/MS analyzed as the quantitative information is unraveled only in the MS/MS spectrum by the reporter ions of the different forms of the iTRAQ label (see [Figure 1-1](#)).

Peptides occurring in all labeled mixtures are identified as pairs (SILE pairs). The area or intensity ratio of the two peaks (e.g. L/H) of a SILE pair is a measure of the change in the peptide abundance in the mixtures. The ratio of the peptide abundances is called regulation.

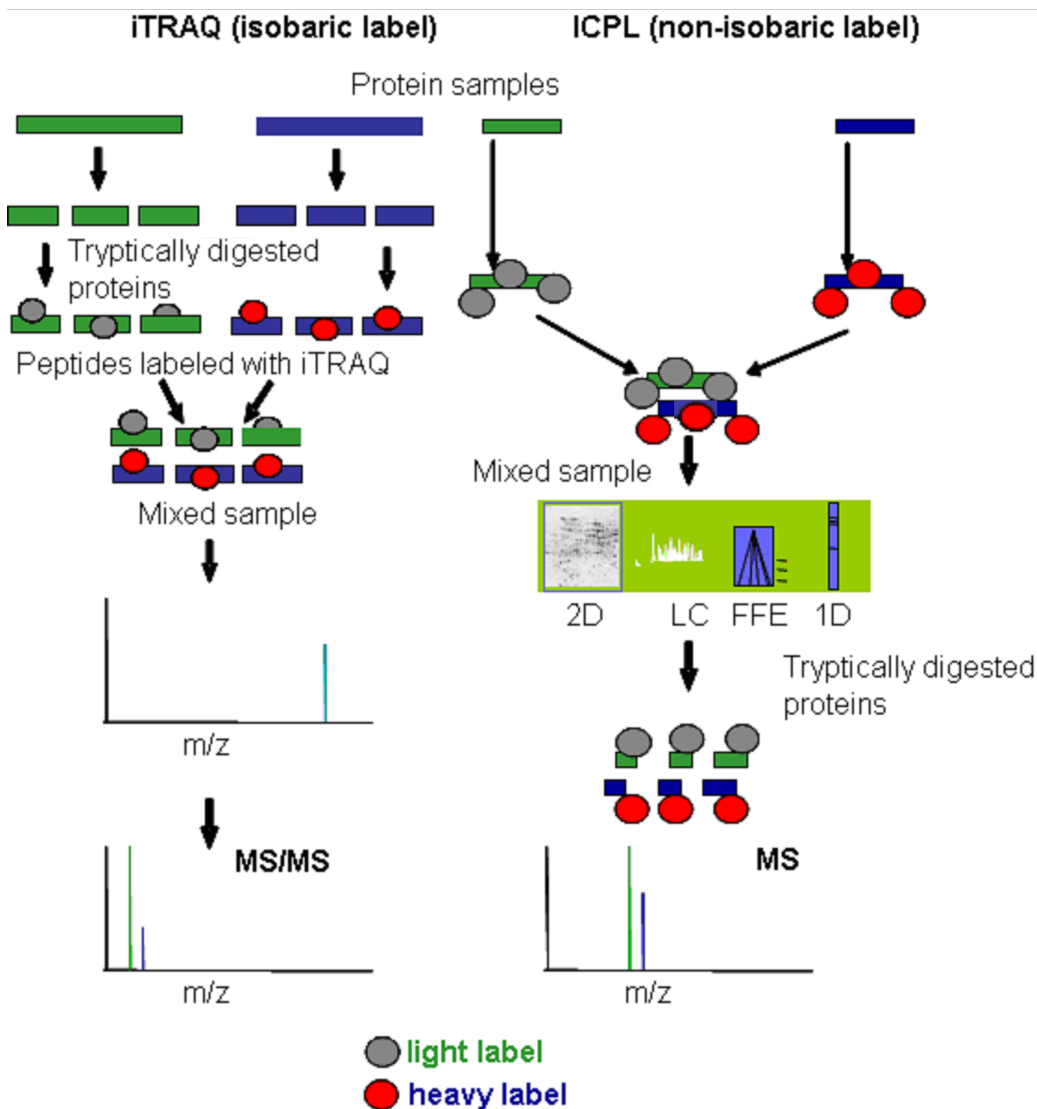


Figure 1-1 Typical workflows for common label chemistries iTRAQ peptides are labeled post-digest, and quantification is performed based on reporter ions in MS/MS spectra. All peptides need to be MS/MS analyzed. ICPL permits protein pre-fractionation because labeling is done prior to protein digests.

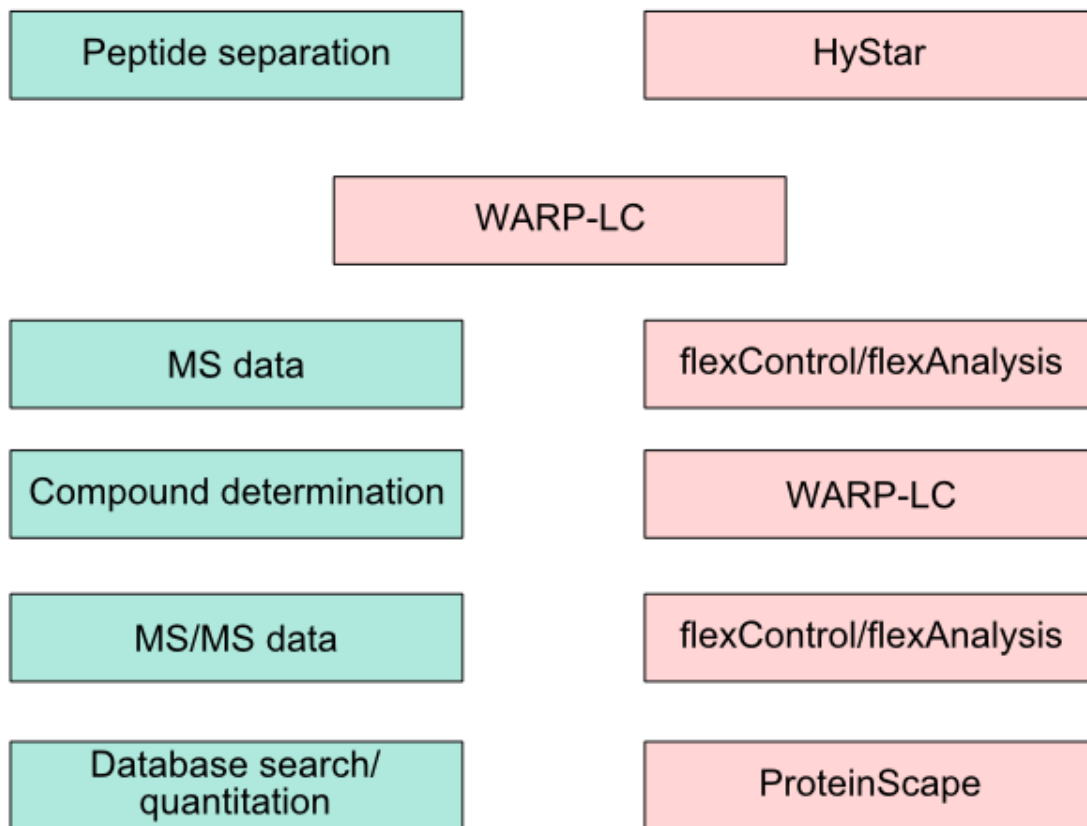


Figure 1-2 The LC-MALDI SILE workflow using WARP-LC

SILE labeled protein digest samples are separated via LC and eluting peptides are directly deposited onto a MALDI-target. The MS- and MS/MS-data acquisition is controlled by flexControl triggered by WARP-LC. As soon as all MS-data are available, WARP-LC determines compounds from all peak lists. MS/MS-data acquisition is triggered by WARP-LC according to MALDI-MS/MS parameters defined in the selected WARP-LC method.

After the MS/MS measurement is finished and all peak lists from flexAnalysis are available, WARP-LC triggers a Mascot search via ProteinScape of the complete data set. Finally, quantitation of the peptides of each identified protein is performed and results are displayed on the "Proteins & Peptides" page of ProteinScape 3.0.

1.2 Tutorial Data

Tutorial Data for this tutorial are provided on the WARP-LC installation DVD. The tutorial data need to be copied to your computer before you can use them. The data are collected in the directory "LC-MALDI-SILE".

Note To use the tutorial data, you need ProteinScape 3.0 and compass 1.3 Patch 5 or higher (flexControl 3.3.108 and flexAnalysis 3.3.80) or compass 1.4. Please refer to manuals and release notes in order to install the programs (maldi.sw.support@bdal.de). Drive d:\ is mandatory.

The path of the tutorial data on your computer should be:

```
D: /data/ Tutorial Data/WARP- LC/ LC- MALDI- SILE/ICPL2plex_
standard_protein_mix.
```

1.3 Sample Preparation

A mixture of 3 proteins (bovine serum albumin, carbonic anhydrase and ubiquitin) was labeled using ICPL™ kit (Serva, Germany) as described in the manual. An aliquot of the tryptic digest was diluted by a factor of 20 and 5 µL of this dilution was used for LC separation.

For the LC-MALDI analysis of ICPL-labeled protein digest mixtures the following **LC conditions** are recommended (used for the data presented in this tutorial):

Easy-nLC	(Bruker) with a flow rate of 0.3 µL/min
Column:	75 µm id.x15cm (PepMap, LC Packings)
Solvent A:	water, 0.05% trifluoroacetic acid
Solvent B:	acetonitrile, 0.05% trifluoroacetic acid
Gradient:	0-48 min 45% B; 48-60 min 95% B; 60-70 min 5% B
Fraction collection:	every 15 sec
MALDI-target:	MTP AnchorChip™ 800/384

A continuous flow of 100 µL/h matrix solution was spotted during fractionation via a syringe pump connected to the capillary coming from the LC resulting in 0.4 µL matrix solution per spot.

Matrix solution: 748 µL 90% Acetonitrile/water

36 µL saturated solution of HCCA in 95% Acetonitrile/ water

8 µL 10 % TFA in water

8 µL 100 mM $\text{NH}_4\text{H}_2\text{PO}_4$ in water

Calibration spots: Peptide calibration mix (Bruker) was dissolved in 125 µL 30 % Acetonitrile/water/0.1 % TFA. 10 µL of this solution was mixed with 90 µL of the matrix solution and 0.5 µL were manually deposited to each calibration anchor on chip 1 of the target.

AutoXecute Method for calibration:

LC-MALDI MScalibrate.axe

AutoXecute Method for MS acquisition:

LC-MALDI ICPL2plex MSmeasure.axe

AutoXecute Method for MS/MS acquisition:

LC-MALDI MSMSmeasure.axe

flexAnalysis Method for calibration:

LC-MALDI MScalibrate.FAMSMMethod

flexAnalysis Method for MS acquisition:

LC-MALDI ICPL2plex MSprocessing.FAMSMMethod

flexAnalysis Method for MS/MS acquisition:

LC-MALDI MSMSprocessing.FALIFTMethod

WARP-LC Method:

LC-MALDI SILE Tutorial.WarpLCMethod

ProteinScape Method
(database search):

LC-MALDI_ICPL2plex_ArgC

The methods are all installed during WARP-LC 1.3 and ProteinScape 3.0 installation. You will also find them on the CD (despite the ProteinScape method).

2 LC-MALDI SILE Workflow

The **LC-MALDI SILE** workflow in WARP-LC supports the MS- and MS/MS-data acquisition of isotopic labeled protein/peptide samples. A SILE labeled sample is LC separated and fractions of the eluting peptides are deposited on a MALDI target. Each sample spot on the MALDI target represents a chromatographic fraction. The MS- and MS/MS-data acquisition of such a prepared LC run requires the definition of an **AutoXecute Run**. Thus, the AutoXecute Run is the central data source for the measurement of the LC-MALDI based workflow.

2.1 WARP-LC: New AutoXecute Run

Start WARP-LC 1.3 and log in ProteinScape 3.0 using your personal account and password (see also ProteinScape 3.0 user's manual). Under **File** you can start a wizard under **New AutoXecute Run** leading you through the different steps of the AutoXecute run definition.

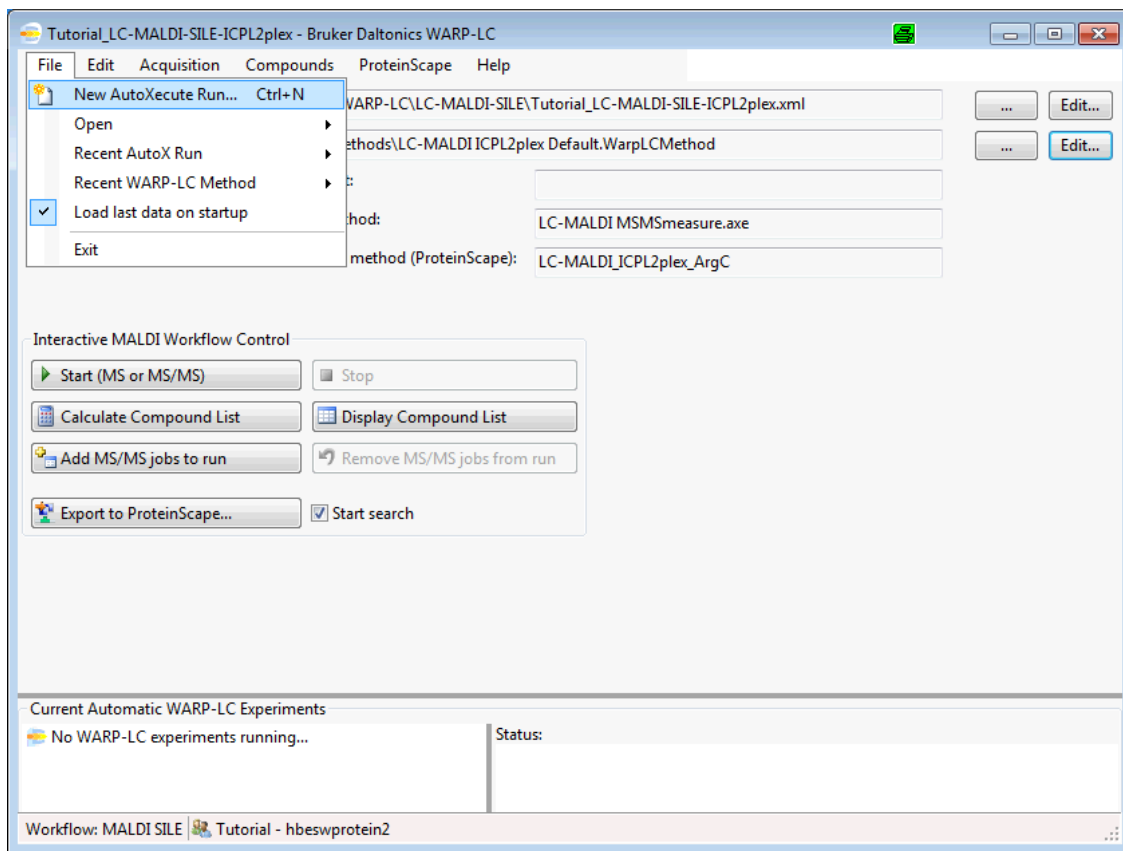


Figure 2-1 Definition of a new AutoXecute Run in WARP-LC. Start the wizard by selecting **File >New AutoXecute Run**.

The next step is the selection of a MALDI sample plate type. For short or medium long LC gradients, an MTP AnchorChip 800-384 is recommended. This sample plates provides 384 sample spots and 96 calibration spots on different chips. For longer LC gradients, MTP AnchorChip 1536 TF is recommended.

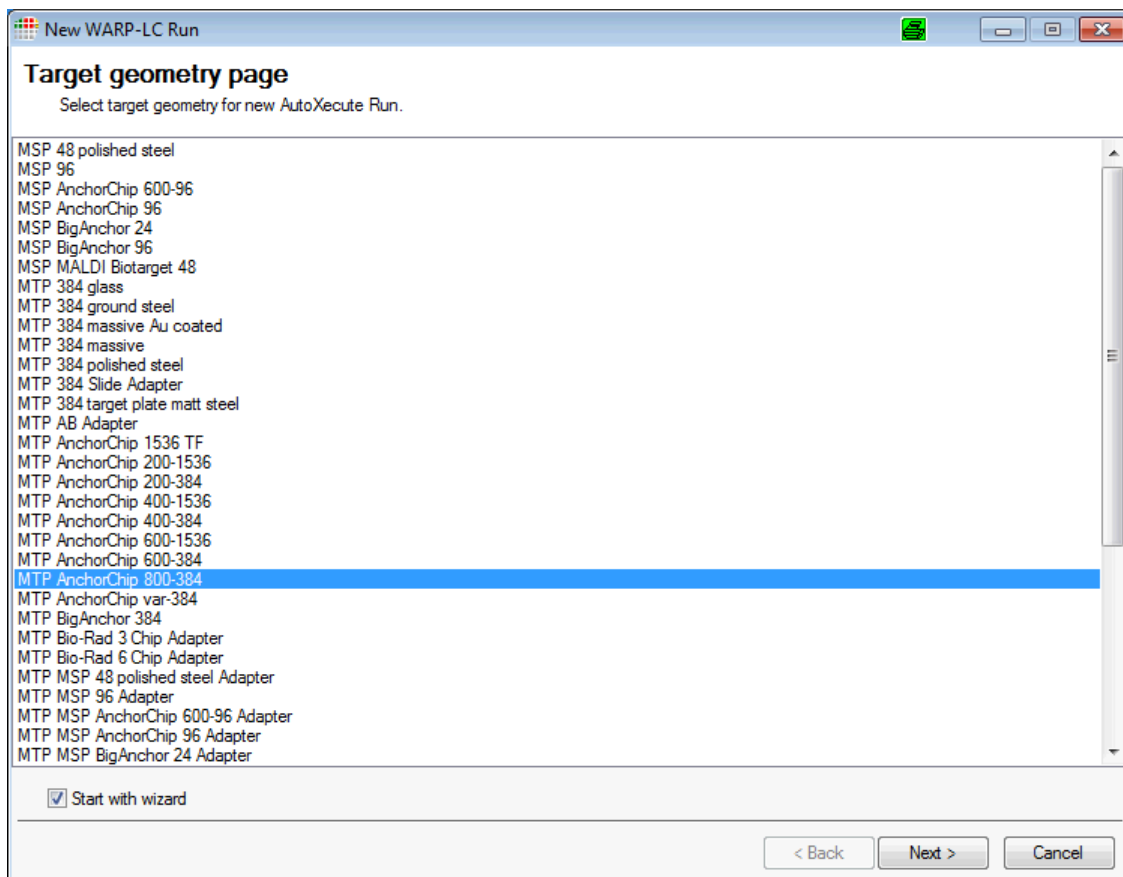


Figure 2-2 **Selection of a MALDI sample plate. Most used and recommended sample plate type for LC-MALDI experiments is the “MTP AnchorChip 800-384”.**

The next step of the wizard is the selection of the sample spots covered by the LC fractions by drag-and-drop.

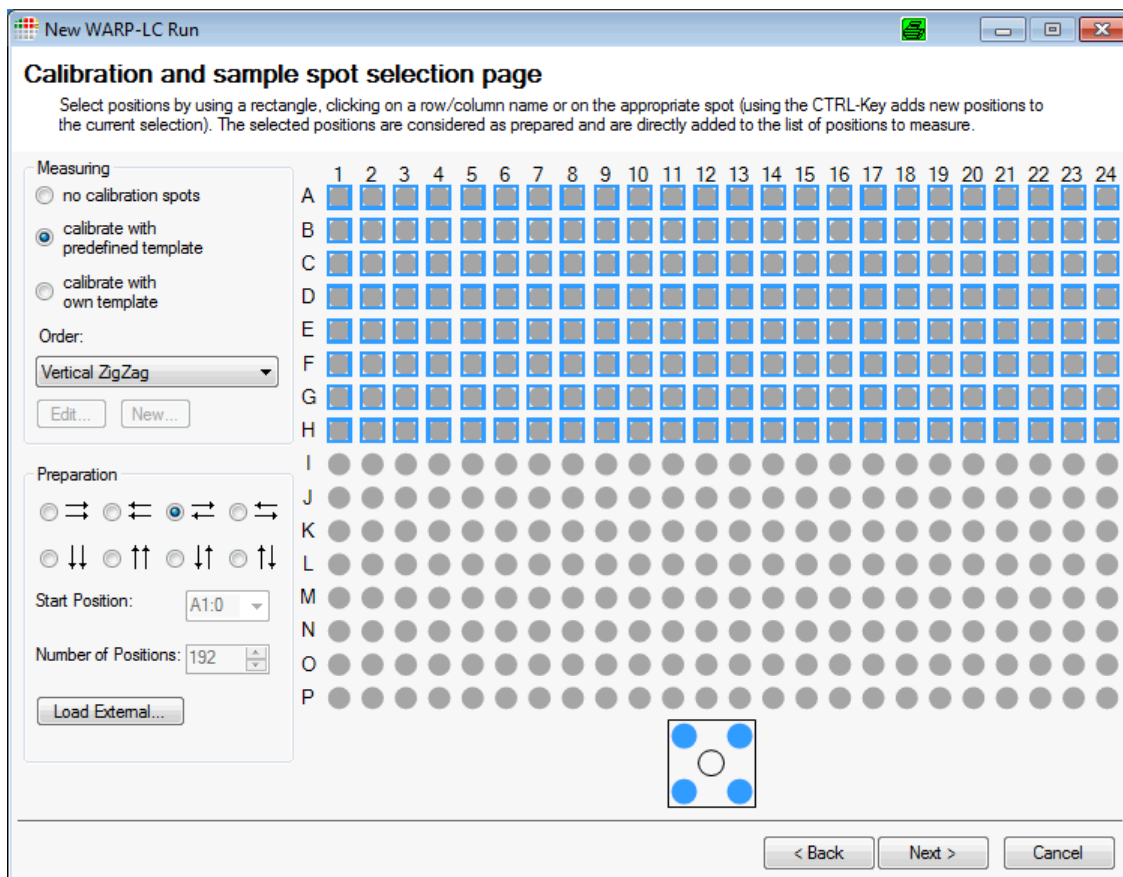


Figure 2-3 Definition of LC-MALDI fractions and preparation/measuring order

Preparation order has to be defined according to LC fractionation order, **measuring order** can be defined independently of preparation order. It is recommended to use “Peptide calibration standard II” (Part-No. 222570, Bruker Daltonics) for external near neighbor calibration.

Run parameter page

Select methods for the measuring positions. If the Calibration mode is activated, the respective methods are automatically assigned to calibration spots. MS and MS/MS methods are assigned to the regular measuring spots. The retention time is calculated based on the previously selected preparation order.

MS

Calibration

☒ Calibration

AutoXecute Method: LC-MALDI MScalibrate Edit

flexAnalysis Method: none

AutoXecute Method: LC-MALDI ICPL2plex MSmeasur Edit

flexAnalysis Method: none

Data Destination

Data Directory: D:\Data\Tutorial Data\WARP-LC

Sample Name: LC-MALDI-SILE

Comments

Comment 1:

Comment 2:

ProteinScope

Experiment ID: 281474976716977

WARP-LC

Method: LC-MALDI SILE Tutorial

ESI Analysis:

Retention time

Delay: 00:25:00 h:min:s Time Slice: 15.00 s

☐ Stop after MS measurement

☐ Clean source after measurement

< Back Next > Cancel

Figure 2-4 Selection of AutoXecute Methods for the LC-MALDI analysis

In the **Run parameter page** (Figure 2-4) of the AutoXecute Run Wizard the AutoXecute Methods for data acquisition of the calibrants and sample spots are defined as well as the data directory and the sample name relevant for storage of the spectra. The recommended data directory is: `d:\data\folder name\`. Additionally, the ProteinScope Experiment ID has to be selected in order to properly load the data into ProteinScope. The WARP-LC method contains important settings for MS/MS acquisition, database search and quantitation

Note The FlexAnalysis Method is defined directly in flexControl/AutoXecute; the ProteinScope-Method and MS/MS-AutoXecute-Method are defined in the WARP-LC Method.

The calculation of the **Retention time** for each fraction is based on:

- a. 'Delay' (between injection of the sample onto the LC column and the start of fraction collection)
- b. the 'Time Slice' used (collection time per fraction).

These values have to be defined by the user either here or before in HyStar (LC and ProteineerFC fraction collector control software).

To enable data export to ProteinScape, a ProteinScape Experiment ID has to be selected.

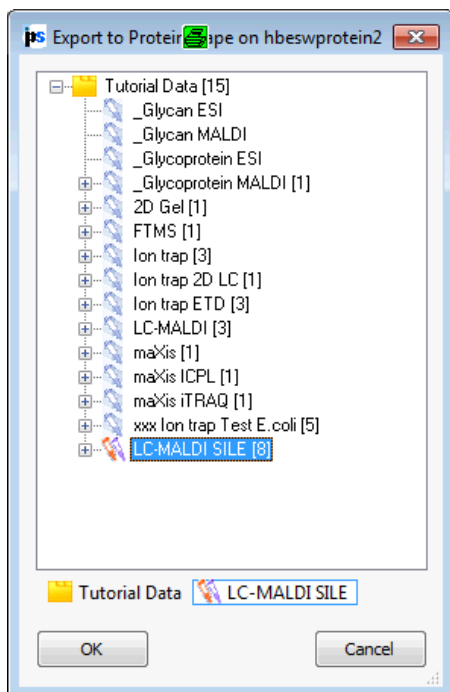


Figure 2-5 Selection of ProteinScape Experiment ID. You have to choose your ProteinScape project and sample.

Note The settings for **Calibration**, **AutoXecute Calibration Method**, **AutoXecute MS Method**, **Data Directory**, **Sample Name**, **Comments**, **WARP-LC Method** and **Retention Time Offset** and **Interval** can be changed before it started.

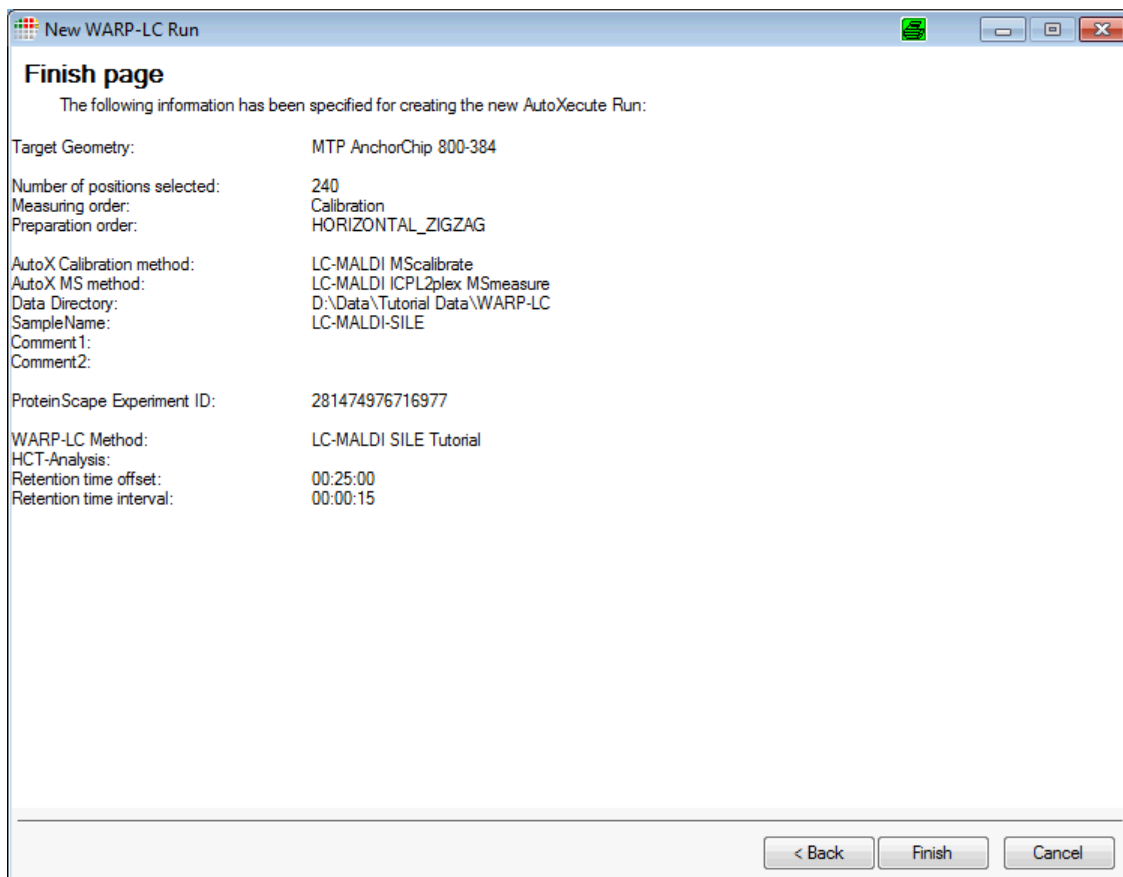


Figure 2-6 Overview of the selected methods and other settings specified for the new AutoXecute Run

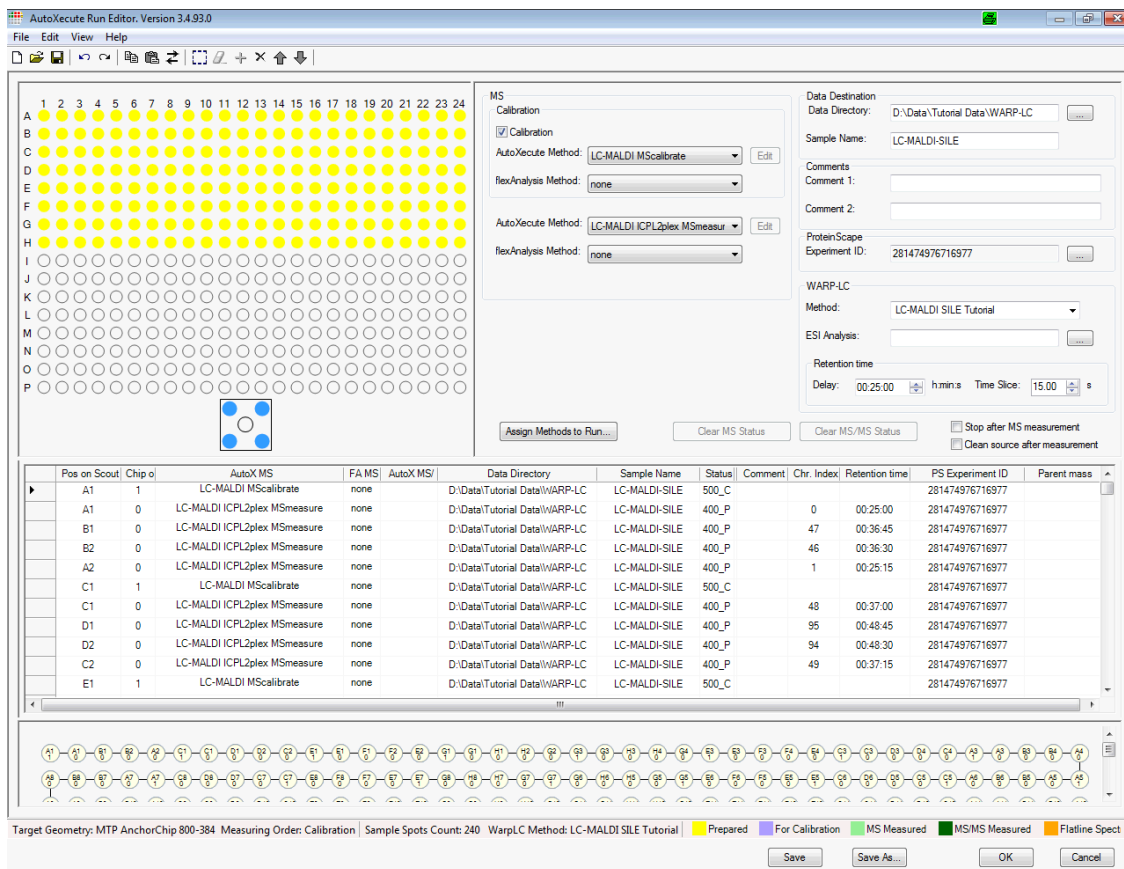


Figure 2-7 Complete overview of the new AutoXecute Run

The change of settings in the AutoXecute Run such as **Methods**, **Data directory**, **Sample Name** or **Retention Time** is possible before the acquisition was started:

1. A different Method is selected or the **DataDirectory**, **Sample Name** or **Comment** is changed.
2. The sample spots for which settings should be changed have to be selected either in the target view or in the spreadsheet.
3. The new settings are assigned to the selected sample spots in the Spreadsheet by pressing the button **Assign Methods to Run**.

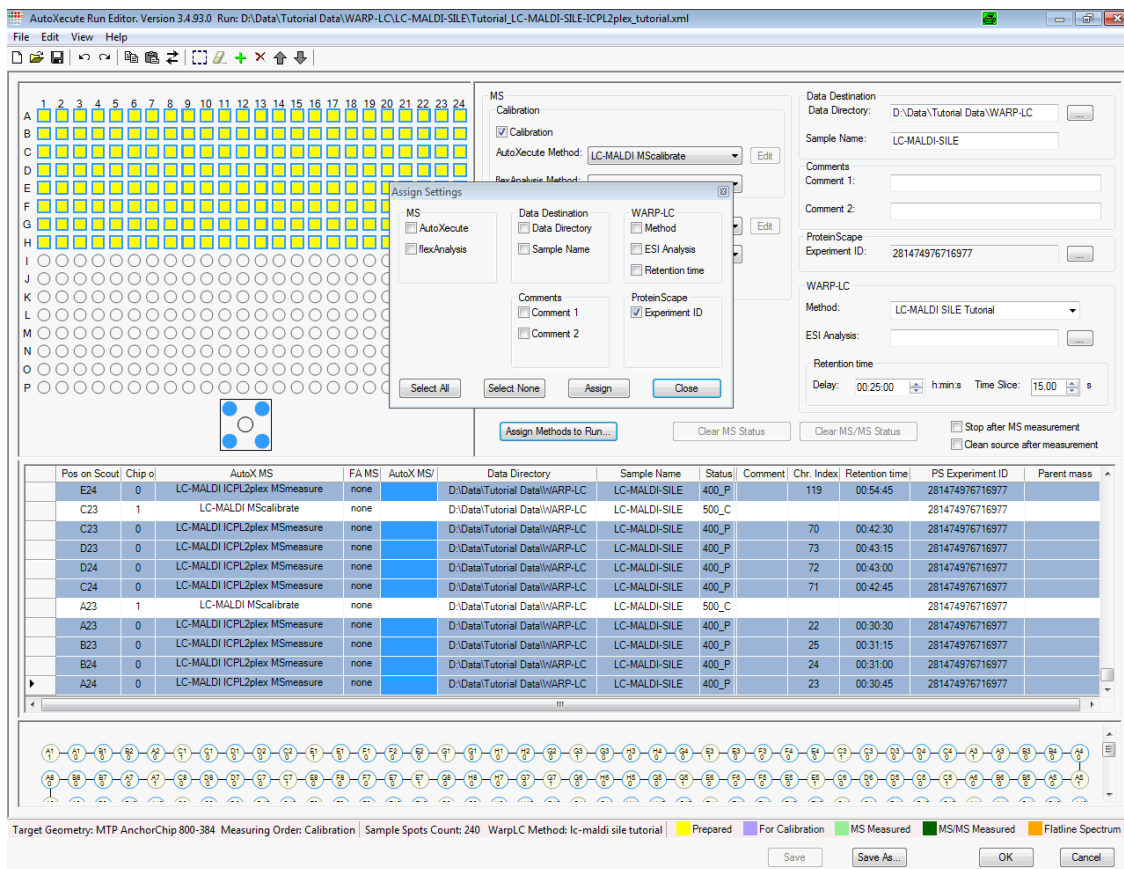


Figure 2-8 Change of settings in the AutoXecute Run (before starting the data acquisition). This example shows how to change or add “ProteinScope Experiment ID” to the run. Select the sample spots, choose new experiment ID and assign the new parameter to the run.

Save the AutoXecute Run either to the default directory d:\Methods\AutoXSequences or directly to the data directory of the analysis.

After saving the AutoXecute Run, the Editor can be closed and the initial screen of the WARP-LC software appears that displays the new AutoXecute Run together with the selected WARP-LC Method.

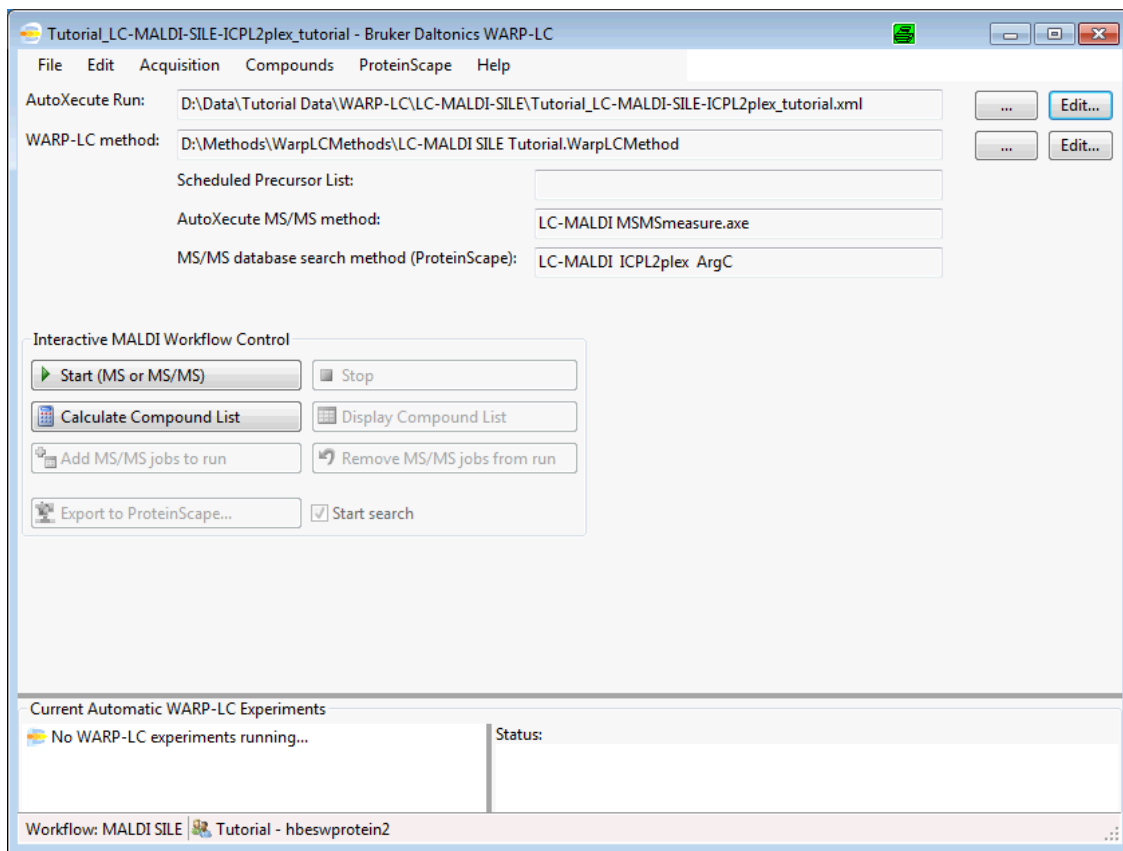


Figure 2-9 AutoXecute run with WARP-LC method in WARP-LC 1.3 before start of the run.

Start the AutoXecute run from the main page of WARP-LC within “Interactive MALDI Workflow Control”. When all the methods are selected properly as described in this tutorial, MS and MS/MS acquisition in flexControl, spectra processing in flexAnalysis and database search and quantitation in ProteinScope will be performed automatically.

Note Before starting the AutoXecute run, FlexControl methods for MS and MSMS acquisition have to be tested. Typically, the methods `RP_700-3200_Da.par` and `Lift.lift` are optimized for LC-MALDI applications. Please check settings like laser power, mass range, detection gain and calibration before starting the analysis!

2.2 AutoXecute Methods

For a complete LC-MALDI analysis **3 different AutoXecute Methods** are required to control the automatic MS- and MS/MS-data acquisition and to perform a proper calibration of the MS. Before you can use the default AutoXecute methods, you should open them and select the flexControl methods valid at your instrument. For calibration and MS measurement, select a flexControl method for MS acquisition in reflectron mode, for MSMS acquisition select a validated Lift method. Save the methods with this selection.

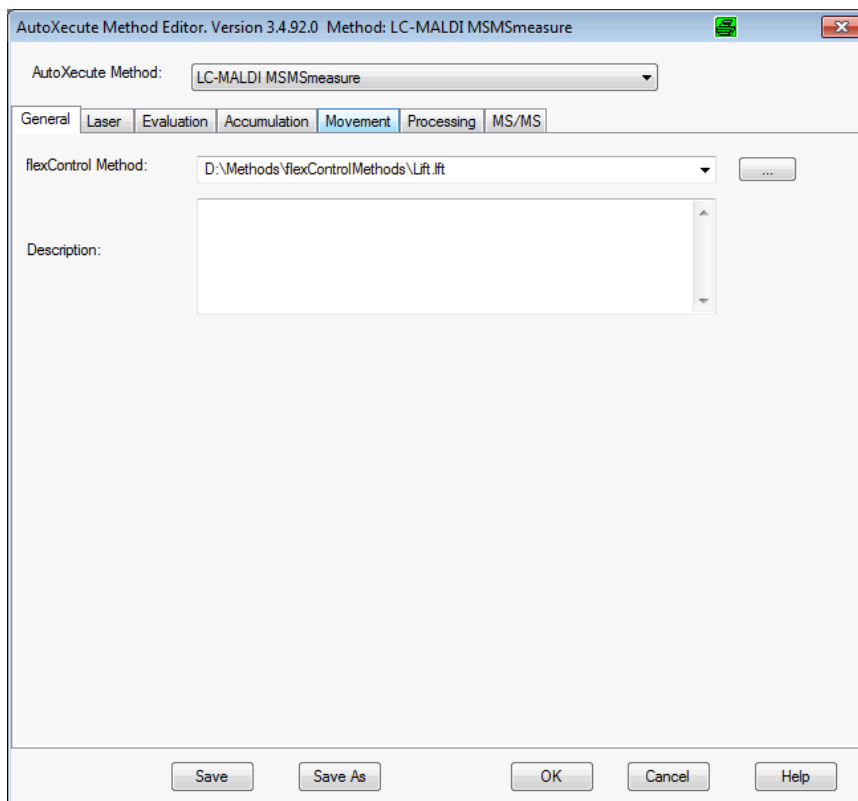


Figure 2-10 Selection of a FlexControl Method

As the **laser power** differs from instrument to instrument, you have to select the proper laser power for each AutoXecute method. Save the methods with this new value.

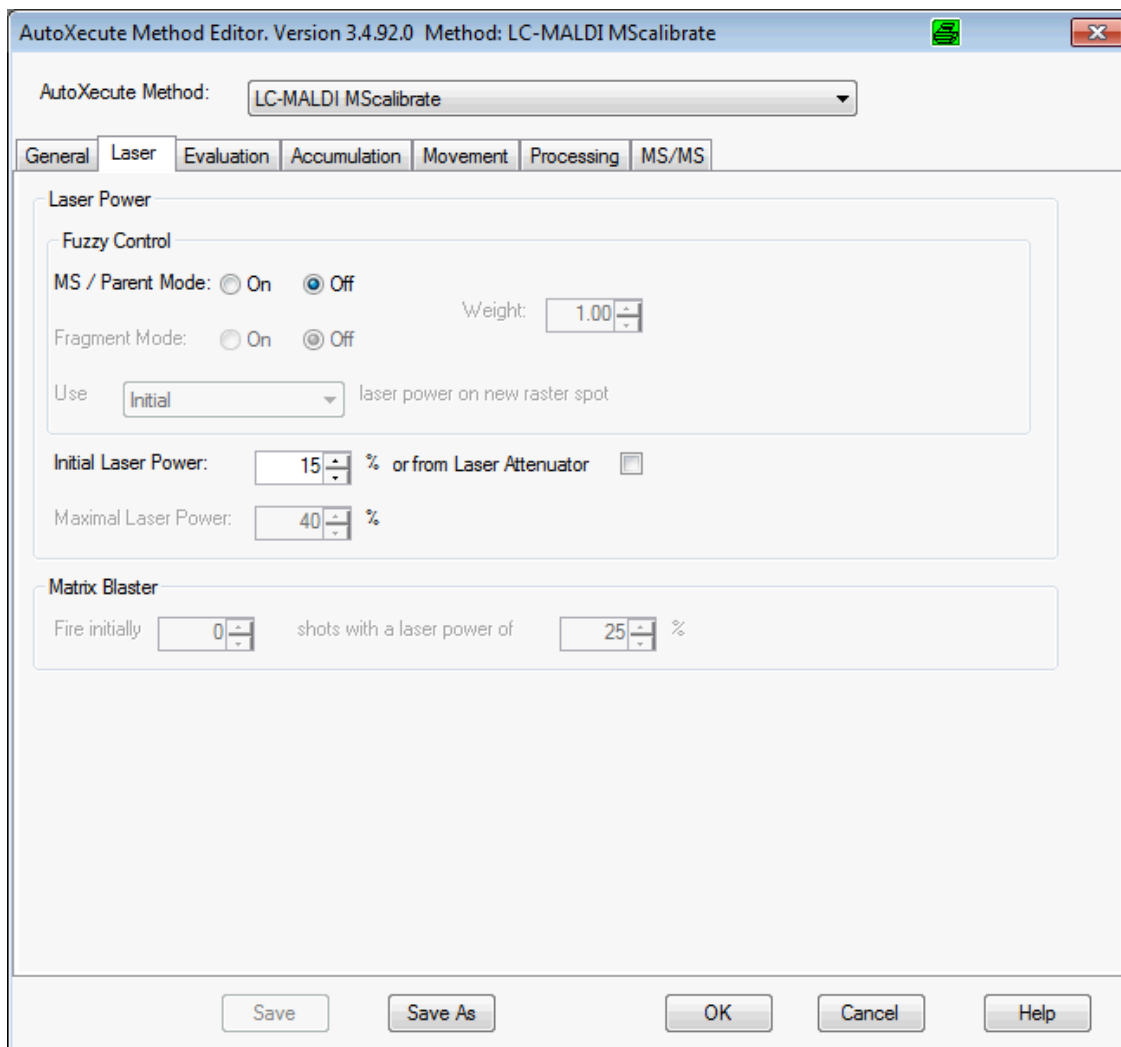


Figure 2-11 Selection of a proper laser power in the AutoXecute methods for MS measurements. This should be done for “LC-MALDI MScalibrate.axe” and for “LC-MALDI MSmeasure.axe”.

Note Before you start an LC-MALDI run, open the AutoXecute methods and edit the laser power. The correct laser power value can be achieved by acquiring MS and MSMS spectra of a typical sample spot. Also find the best laser power value for the calibration spots.

Note The laser power for MSMS acquisition should be optimized directly in the flexControl method! Open your valid *name.lft*-method, optimize laser power for **Parent** and **Fragments** using your sample and save the method.

Every AutoXecute method refers to a specific default **processing method** in flexAnalysis.

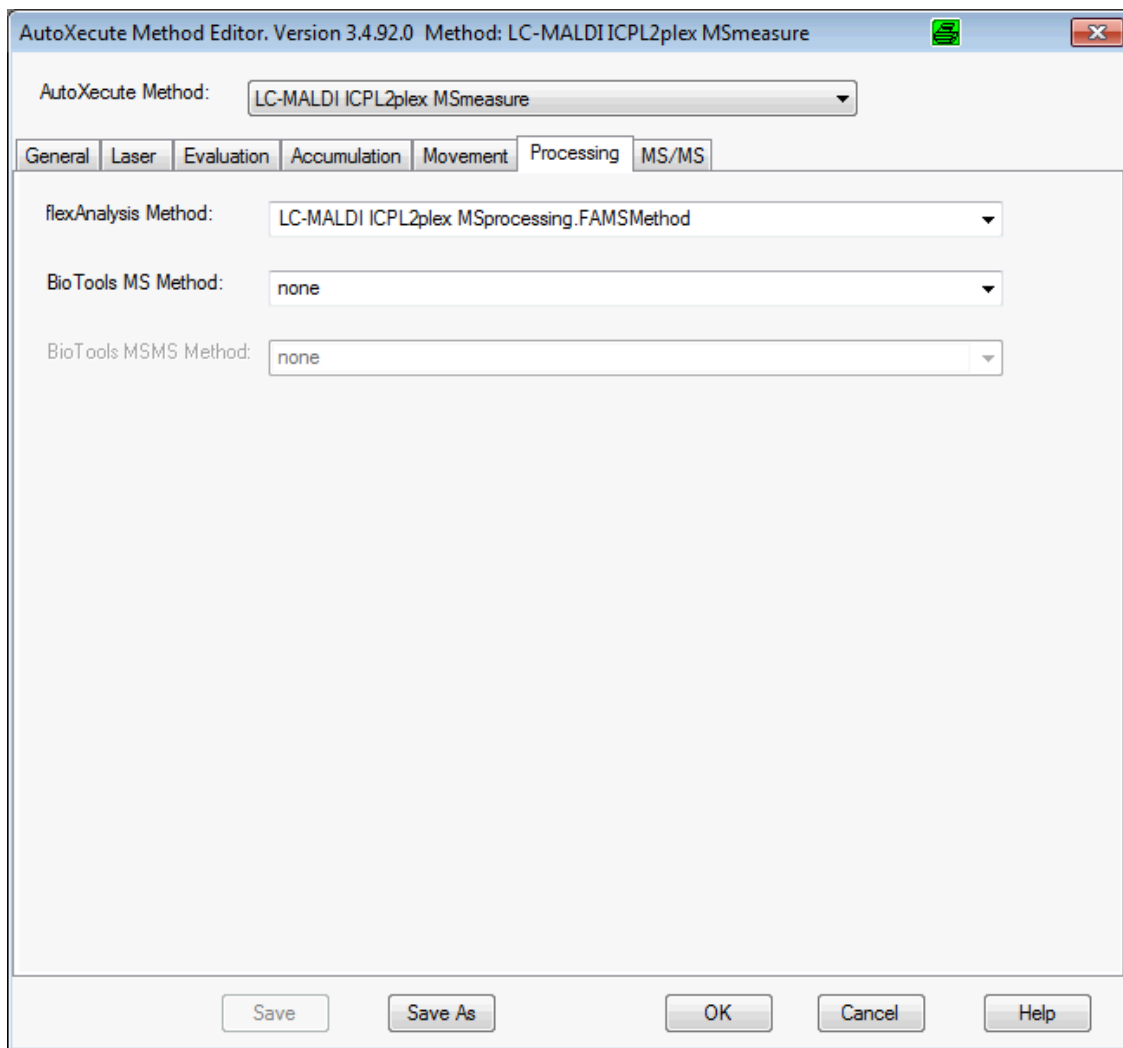


Figure 2-12 Reference to processing method in flexAnalysis

2.3 FlexAnalysis Method

The **flexAnalysis methods** define all required parameter for the MS and MS/MS data processing including peak picking of MS and MS/MS data and calibration of MS data. In the case of ICPL labeled samples, peak picking of the SILE pairs is critical. The specific **flexAnalysis methods** `LC-MALDI ICPL2plex MSprocessing.FAMethod` and `LC-MALDI ICPL4plex MSprocessing.FAMethod` contain specific parameter enabling the peak picking algorithm to properly annotate the SILE pairs.

If you want to check or change parameters of the processing methods, open the method in flexAnalysis and press **Edit Parameters**. For more details, please read the *flexAnalysis User Manual*.

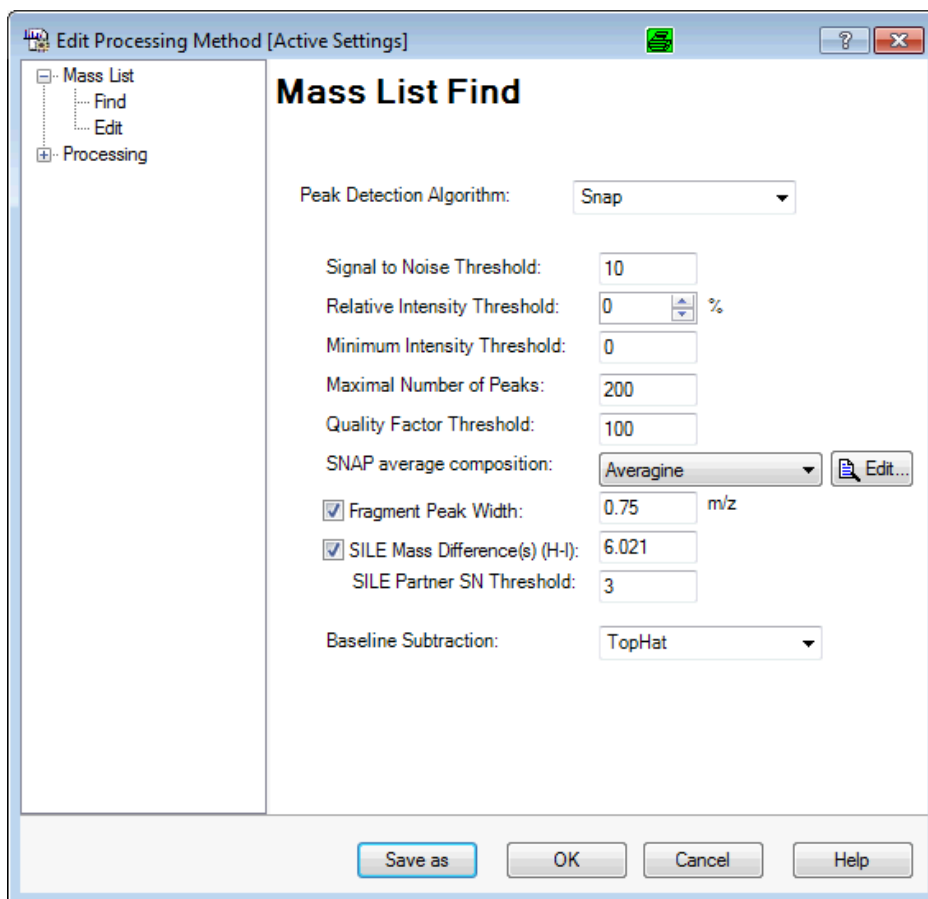


Figure 2-13 FlexAnalysis method for ICPL2plex processing. Expected mass difference of the SILE partners is defined.

2.4 WARP-LC Method

The **WARP-LC Method** defines all required parameters for the MS and MS/MS data acquisition of a chromatographic separated sample. After the MS data acquisition of all fractions, the WARP-LC Method controls the generation of a **non-redundant list of chromatographically separated precursors (compounds)** according to the settings in the WARP-LC Method. This list is called **compound list**. In addition, parameters for a Mascot database search are referenced in the WARP-LC method, which automatically triggers the database search of all acquired MS/MS data.

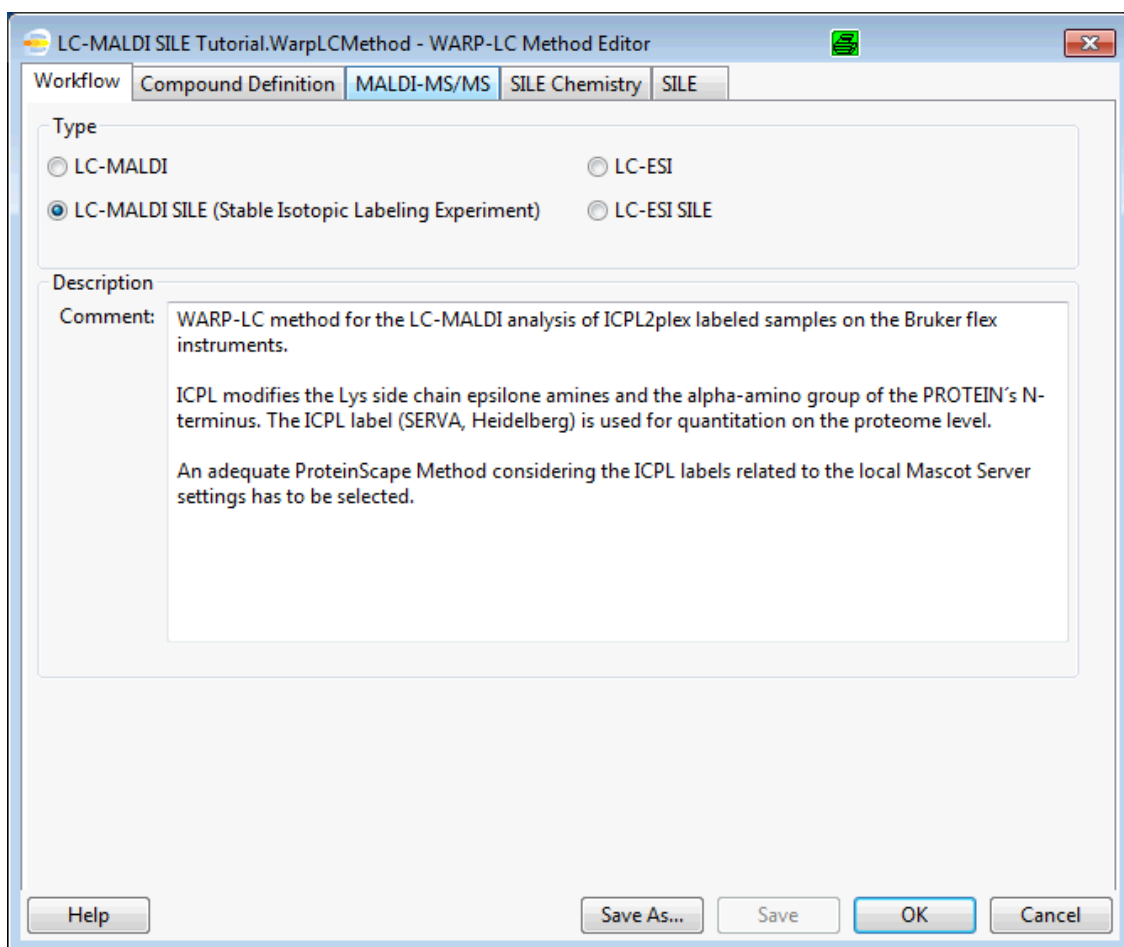
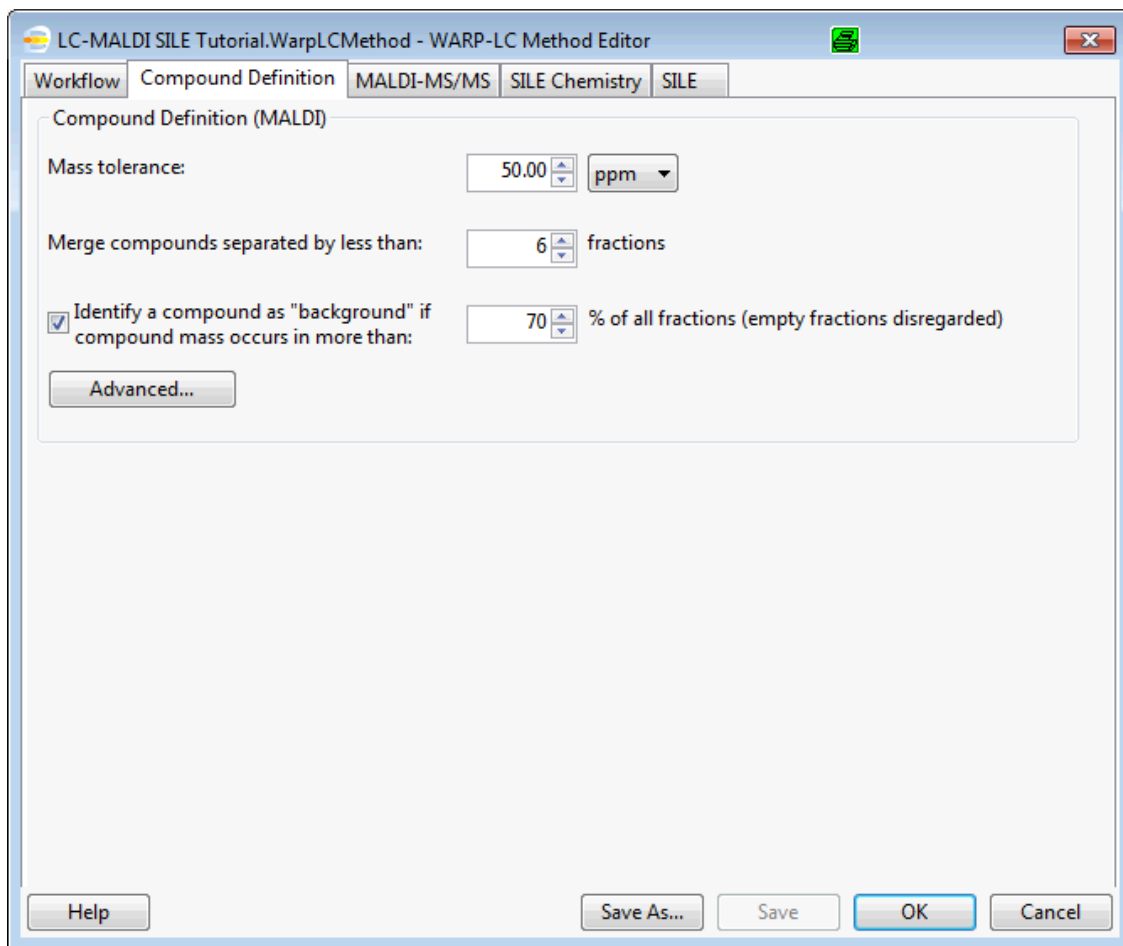


Figure 2-14 Workflow description of LC-MALDI SILE Tutorial.WarpLCMethod.

The compound definition is mainly depending on the **mass accuracy** of your instrument. **Background peaks** can be detected and excluded from the list of scheduled MS\MS precursors.

**Figure 2-15 Compound definition: the mass tolerance for compound calculation is set to 50 ppm. Background peaks are not considered for MSMS acquisition!**

For successful **MSMS acquisition**, a valid AutoXecute method has to be selected. The default method for LC-MALDI experiments is "LC-MALDI MSMSmeasure.axe".

S/N threshold of 10 limits the **selection of precursors** to those with a minimum S/N value of 10.

Additionally, co-eluting compounds and maximum number of MS/MS spectra acquired per fraction/spot are taken into account when scheduling MS/MS applications.

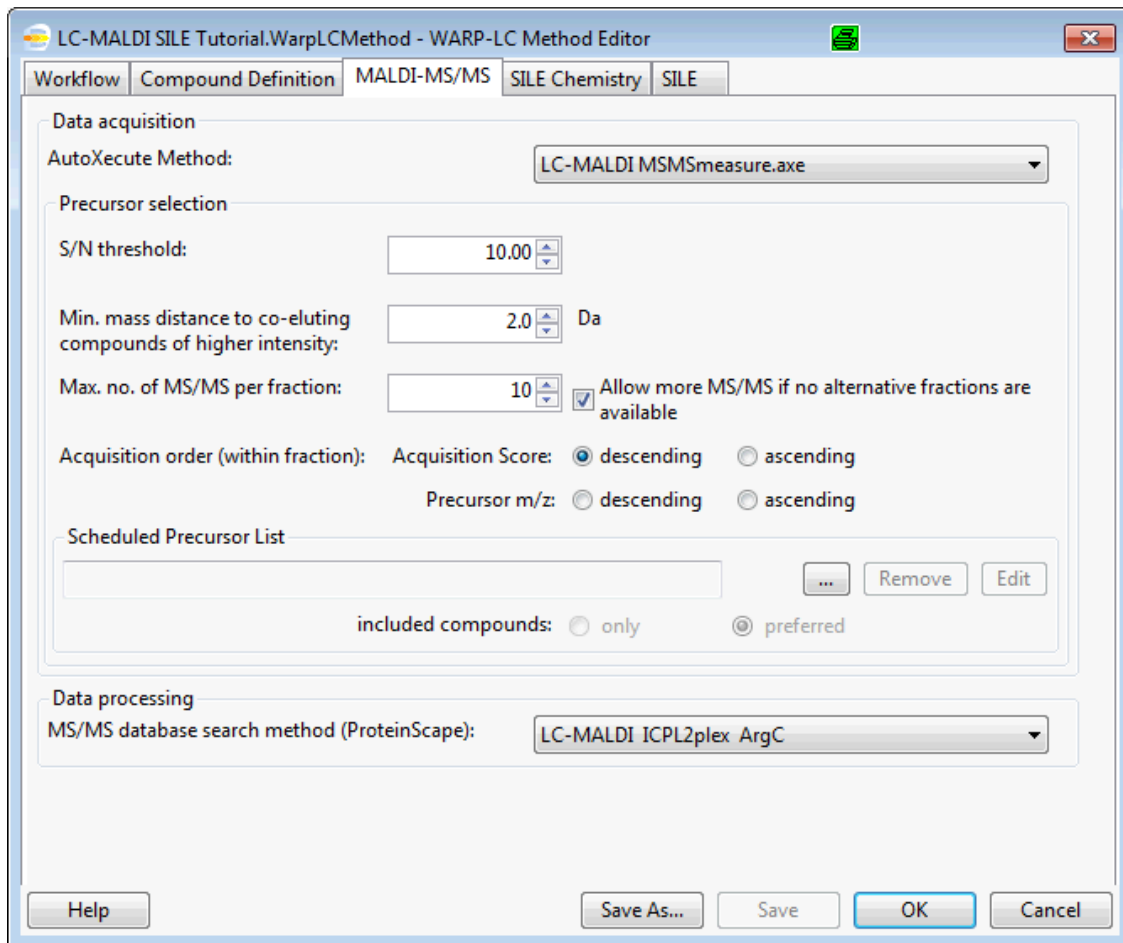


Figure 2-16 The parameter settings for MS/MS acquisition are pooled on the MALDI-MS/MS page

Database searches are performed using ProteinScape 3.0. A proper search method has to be defined there.

Protein Searches
Search Parameter Configuration (MS/MS (PFF))

General Settings

Select search method: LC-MALDI_ICPL2plex_ArgC

Method name: LC-MALDI_ICPL2plex_ArgC

Version: 1.0

Submit: All spectra

Search engines: ☒ Mascot

Precursor: ☐ Use peptide Mr from glycopeptide classification

Max. no. of listed proteins:

Protein list compilation: ☐ By search engine ☒ By ProteinExtractor

☒ Assessment

☐ Second Round

[Configure Rights...](#)

[Save](#) [Save As...](#)

Mascot

Database: SwissProt

Taxonomy: Other mammalia

Enzyme: Arg-C

Allow up to 1 missed cleavages

Modifications:

- Acetyl (K)
- Acetyl (N-term)
- Acetyl (Protein N-term)
- Amidated (C-term)
- Amidated (Protein C-term)

Carbamidomethyl (C): ☒ Fixed ☐ Variable

ICPL (K): ☐ Fixed ☒ Variable

ICPL13C(6) (K): ☐ Fixed ☒ Variable

Oxidation (M): ☐ Fixed ☒ Variable

Peptide tol. $\pm$: 30.0 ppm # ^{12}C : 0

MS/MS tol. $\pm$: 0.8 Da

Peptide charge: 1+ ☒ Monoisotopic ☐ Average

Instrument type CID: MALDI-TOF-TOF

Instrument type ETD: ETD-TRAP

☐ Peptide Decoy (Mascot) ☐ Use Percolator

[? < Back](#) [Next >](#) [Start](#) [Cancel](#)

Figure 2-17 Database search parameter for SILE samples labeled with ICPL2plex (Serva). The method is part of the ProteinScope default setup!

Note The ICPL modifications must be set to **variable**! Although digestion was performed with trypsin, cleavage only occurred at Arginine residues because all Lysines were labeled! Therefore, the best suitable enzyme is **ArgC**.

For **SILE quantitation**, the biological states have to be addressed and the regulation ratio has to be defined.

LC-MALDI SILE Tutorial.WarpLCMethod - WARP-LC Method Editor

Workflow | Compound Definition | MALDI-MS/MS | SILE Chemistry | SILE

MS/MS database search method (ProteinScape): LC-MALDI ICPL2plex ArgC

Isotope Purity Correction...

Biological Experiment

Biological State
ICPL
ICPL:13C(6)

Labels assigned to Biological States

Label	Elemental Formula	Mono-Isotopic Mass	Biological State
ICPL	H(3) C(6) N(1) O(1)	105.021464	ICPL
ICPL:13C(6)	H(3) 13C(6) N(1) O(1)	111.041593	ICPL:13C(6)

Reported Ratios

Ratio Name	Numerator (x/)	Denominator (/y)
L/H	ICPL	ICPL:13C(6)

Help Save As... Save OK Cancel

Figure 2-18 SILE chemistry is selected according to modifications defined in the ProteinScape search method LC-MALDI_ICPL_2plex_ArgC. Biological states can be defined according to individual projects.

SILE quantitation can be performed on **peak area** values or on **peak intensities**. Additionally, the mass tolerance of label recognition can be set here.

Depending on the label chemistry, peptides of same sequence but with different isotopic labels show slightly different retention time. This is e.g. the case for ICPL 4plex labeled samples. To address this phenomenon, **Coeluting Isoforms** should be unchecked and the retention time tolerance of 2 peptides should be defined.

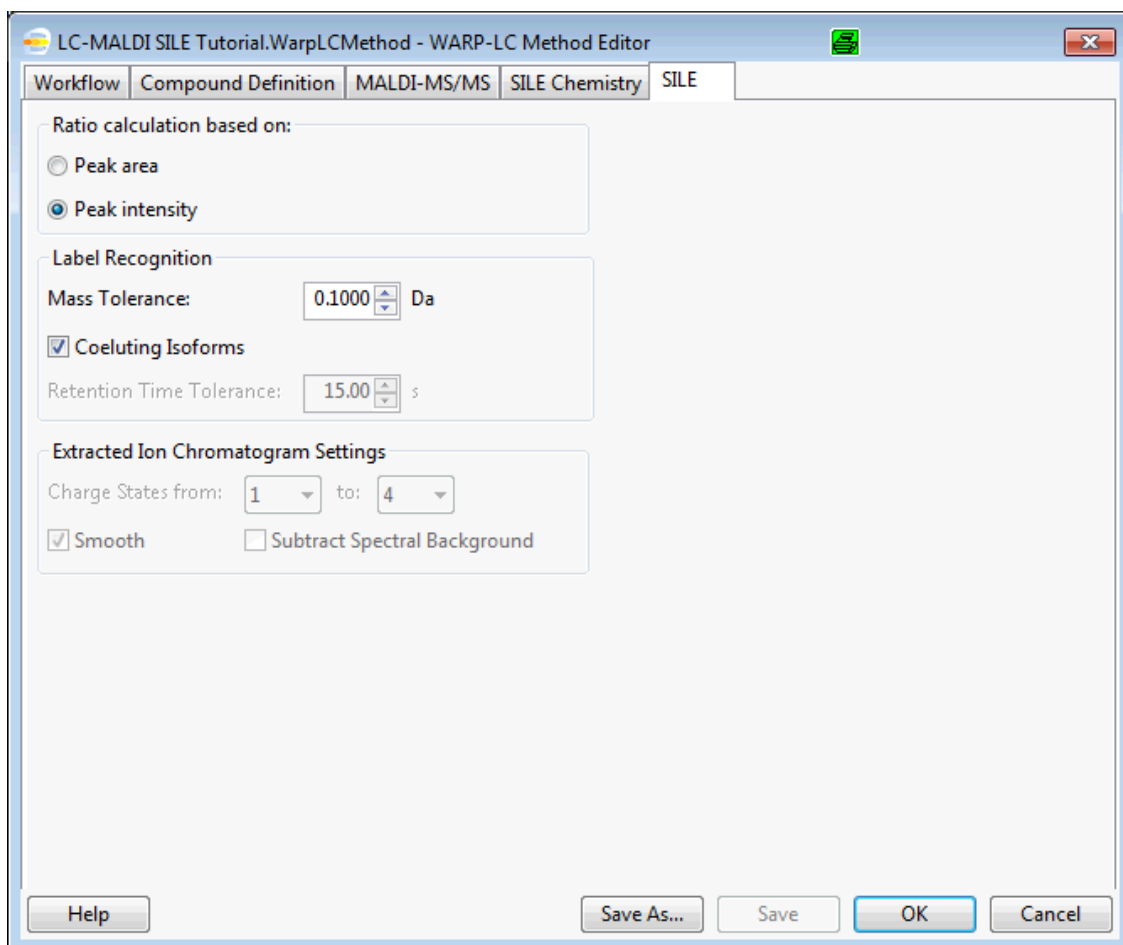


Figure 2-19 Parameter settings for SILE quantitation.

3 ProteinScape

After MS and MSMS acquisition has finished, the data are automatically sent to ProteinScape 3.0. In the case, no ProteinScape experiment number was selected in the AutoXecute run, the data export can be triggered manually from WARP-LC.

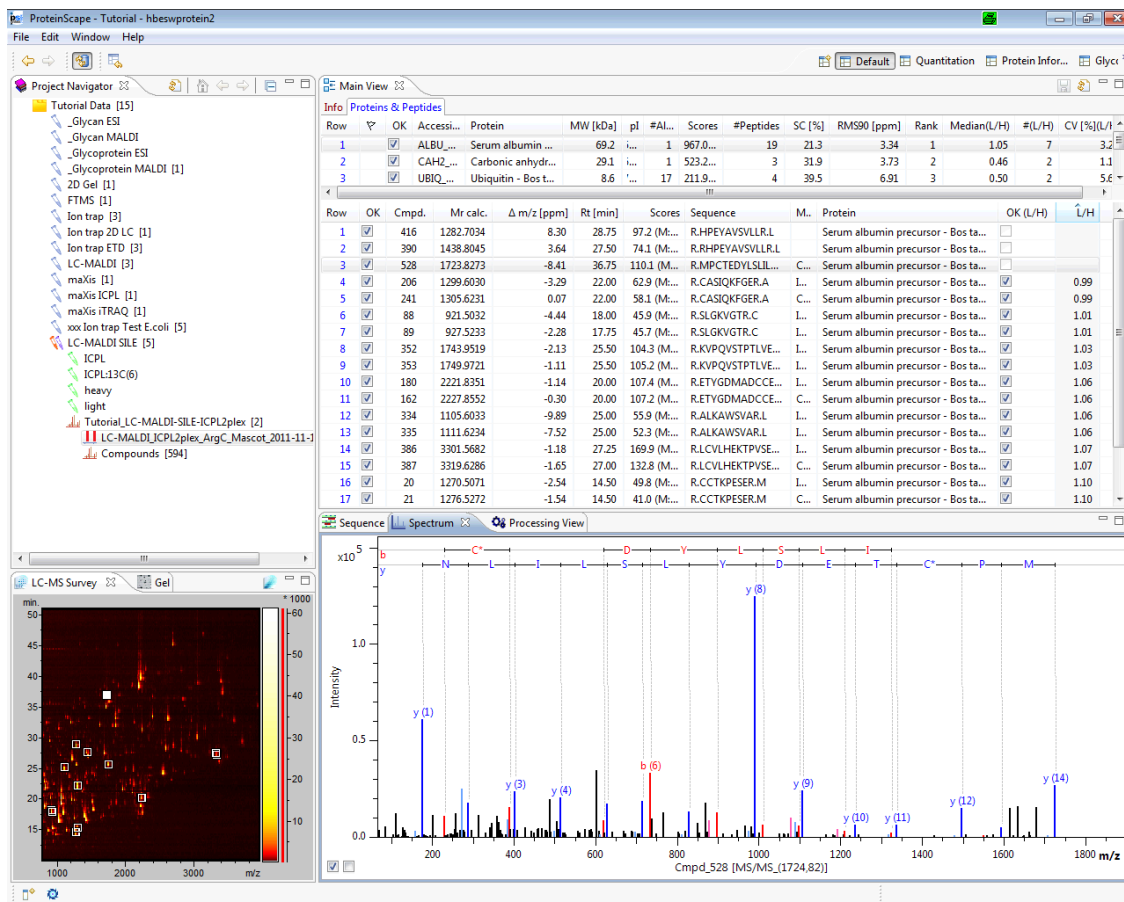


Figure 3-1 Database search and quantitation results in ProteinScape 3.0. The first protein in the list, serum albumin, was selected. The peptide list of serum albumin was sorted on L/H quantitation results.

Results: 3 proteins have been identified and ratios of ICPL light / ICPL heavy (L/H) labeled samples have been calculated.

For bovine serum albumin, the theoretical value of L/H is 1.0, the calculated is 1.05 (CV of 3.2 %). The theoretical L/H values of carbonic anhydrase and ubiquitin were 0.5, the experimental value were 0.46 and 0.5 respectively.

Protein	Regulation (L/H) experimental	Regulation (L/H) theoretical
Serum albumin	1.05 (median)	1.0
Carbonic anhydrase	0.46 (median)	0.5
Ubiquitin	0.50 (median)	0.5

When the database search is started by the user from ProteinScape and not from WARP-LC, the SILE-quantitation is not triggered automatically. After the search is finished, go to page “Info” and start the SILE quantitation.

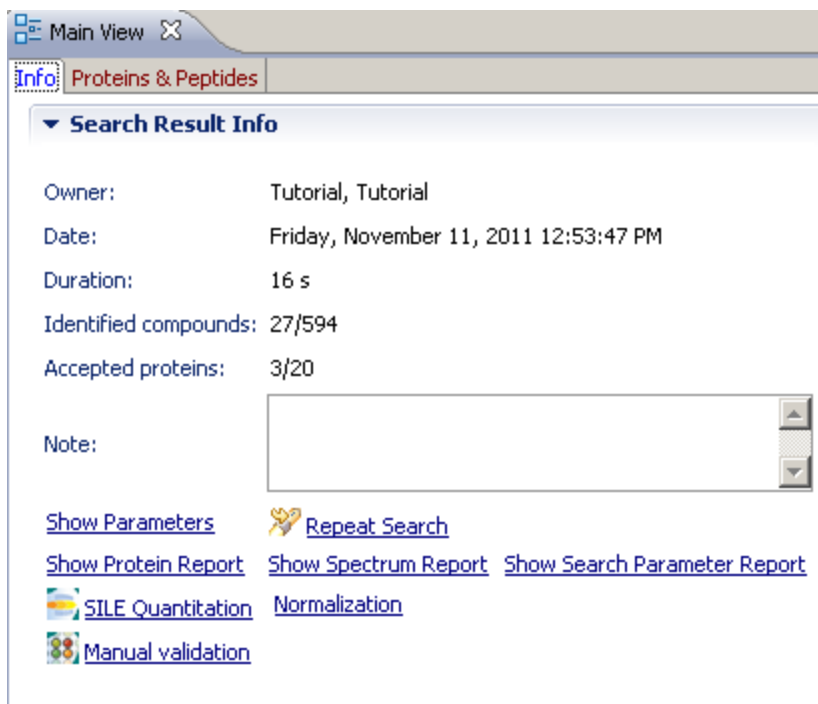


Figure 3-2 Press SILE Quantitation on the Info page to start quantitation in WARP-LC. Important: a WARP-LC method for ICLP SILE has to be assigned to the run in WARP-LC!